



Antigens of the type-three secretion system of *Aeromonas salmonicida* subsp. *salmonicida* prevent protective immunity in rainbow trout[☆]



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ABSTRACT

Aeromonas salmonicida subsp. *salmonicida* is the etiologic agent of furunculosis, a frequent and significant disease of fisheries worldwide. The disease is largely controlled by commercial oil adjuvanted vaccines containing bacterins. However, the mechanisms leading to a protective immune response remain poorly understood. The type-three secretion system (T3SS) plays a central role in virulence of *A. salmonicida* subsp. *salmonicida* and thus may have an influence on the immune response of the host. The aim of this study was to evaluate the role of the T3SS antigens in mounting a protective immune response against furunculosis.

Rainbow trout were intraperitoneally vaccinated in two independent experiments with bacterins prepared from a wild-type *A. salmonicida* strain and an isogenic strain carrying a deletion in the T3SS (Δ ascV). Fish were challenged with the wt strain eight weeks after vaccination. In both trials, the survival rate of trout vaccinated with the Δ ascV strain was significantly higher (23–28%) in comparison to the group vaccinated with the wt strain. High-throughput proteomics analysis of whole bacteria showed the ascV deletion in the mutant strain resulted in lower expression of all the components of the T3SS, several of which have a potential immunosuppressive activity. In a third experiment, fish were vaccinated with recombinant AcrV (homologous to the protective antigen LcrV of *Yersinia*) or S-layer protein VapA (control). AcrV vaccinated fish were not protected against a challenge while fish vaccinated with VapA were partially protected.

The presence of T3SS proteins in the vaccine preparations decreased the level of protection against *A. salmonicida* infection and that AcrV was not a protective antigen. These results challenge the hypothesis that mounting specific antibodies against T3SS proteins should bring better protection to fish and demonstrate that further investigations are needed to better understand the mechanisms underlying effective immune responses against *A. salmonicida* infection.

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1. Introduction

The Gram-negative bacterium *Aeromonas salmonicida* subsp. *salmonicida* (hereafter referred to as *A. salmonicida*) is the etiologic agent of furunculosis. This disease, first described by Lehmann and Neumann in 1896 [1], can affect all salmonid species and is a frequent and significant pathogen of fisheries worldwide. Furunculosis results in marked-economic losses and drives the intensive use of antibiotics [2]. It has been more than 70 years since the first protective vaccination trials against furunculosis were reported [3]. For the past 20 years, the disease has been largely controlled by the use of protective commercialized oil-adjuvanted vaccines containing *A. salmonicida* bacterins [4]. Numerous trials have been performed in order to optimize vaccination and to elucidate the specific antigens involved in protection. Results however,

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have been contradictory and certain antigens capable of producing agglutinating antibodies against *A. salmonicida* were not protective [5–7]. Furthermore, several authors have suggested that the protection observed was, in part non-specific [8]. In contrast, passive immunization of fish with immune sera directed against whole *A. salmonicida* antigens have shown that humoral factors were able to protect fish [9,10] and others have found that the level of protection correlated with the level of specific antibodies in the serum [11,12]. A certain degree of protection with specific antigens of *A. salmonicida* (such as VapA) [13] has been obtained but the immunological mechanisms leading to a protective immune response remains poorly understood while the effect of AcrV in protective immunity has not been studied yet.

Ten years ago, our laboratory published the first descriptions of the type-three secretion system (T3SS) in the *Aeromonas* genus and demonstrated its role as a virulence factor of *A. salmonicida* [14–16]. The T3SS is, in essence, a nanosyringe composed of inner- and outer membrane rings, a needle with a tip and the translocon that injects virulent effector proteins directly into the cytoplasm of the host cell. Despite the prominent role of the T3SS in the pathogenesis of *A. salmonicida* [16–18], knockout mutants of individual T3SS effector genes only partially reduce virulence or delay the onset of the disease after challenge [17]. The aim of the present work was to characterize the protective effect of the T3SS on immunized fish by vaccinating rainbow trout with a formalin-killed wt *A. salmonicida* strain and an isogenic Δ ascV mutant, characterized as T3SS-deficient.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterins were prepared from the following *A. salmonicida* subsp. *salmonicida* strains: wt strain JF5054 (isolated from a trout after experimental infection with strain JF2267 [16]), an Δ ascV mutant (JF2747) and strain JF3239 (Δ ascV/ascV⁺) in which the ascV deletion is complemented *in trans* [18,19]. The wt strain JF2267 was isolated from an arctic char (*Salvelinus alpinus*) and is virulent with intraperitoneal inoculation of 500 colony-forming units (CFU) per fish, sufficient to induce 70–80% of mortality in challenge assays. The isogenic Δ ascV mutant strain was shown to be non-virulent since 10⁵ CFU per fish induced no mortality [18]. Each strain was grown in TSB medium or onto TSA plates at 18 °C with the appropriate antibiotics at the following final concentrations: kanamycin (40 µg/mL) and ampicillin (100 µg/mL).

2.2. Fish

All animals used in this study have been treated according to the Swiss regulations for animal welfare.

The initial bacterin vaccination experiment utilized rainbow trout (*Onchorhynchus mykiss*) of 11 ± 2 cm length and 17 ± 1.5 g weight. Ten fish were transferred to each of six 30 L glass aquaria equipped with a tap water flow through system and aeration. Temperature was maintained at 17.6 ± 0.4 °C. Fish were fed a commercial trout diet at a rate of 1.5% of the body weight; the diet rate was adjusted weekly.

In the second experiment, initial weight of the fish was 23 ± 1.5 g and length was 12 ± 1 cm. Ten fish were placed in each of eight 30 L glass tanks. Temperature was maintained at 15 ± 0.8 °C. Fish were fed commercial diet at a rate of 2% of the body weight; the diet rate was adjusted weekly.

Vaccination with purified proteins was carried out with trout of mean weight 1.5 g. Eighty fish were placed in each of four 130 L

glass tanks. Temperature was maintained at 16 ± 1 °C. Fish were fed commercial diet at a rate of 2.5% of the body weight; the diet rate was adjusted weekly.

2.3. Vaccine preparation

For the preparation of bacterins, 10⁹ bacteria of each strain were inoculated in 50 mL of TSB medium and cultivated overnight at 18 °C with aeration (160 rpm). Bacterial growth was stopped during exponential growth (optical density at 600 nm (OD₆₀₀ = ~1.5). Bacterial suspensions containing 2 × 10⁹ CFU were harvested by centrifugation and inactivated with 1.5% formaldehyde for 2 h at 20 °C under gentle shaking; inactivation of the bacteria was confirmed by cultivation on TSA plates. Suspensions were washed once with PBS pH 7.4 and then resuspended in PBS to obtain a bacterial concentration of 4 × 10⁸ bacterins/mL. This suspension was mixed with 1:1 volume of aluminium hydroxide gel (Sigma) before vaccination and fish were inoculated with 10⁷ bacterins per 50 µL of vaccine solution.

Recombinant AcrV (rAcrV) was expressed in *E. coli* following cloning of the wt *acrV* gene in the pETHis-1 vector [20]. Purification of the protein was carried out by Ni²⁺ chelation chromatography [21].

VapA was prepared directly from the wt *A. salmonicida* strain JF2267. Briefly, bacteria were cultivated in TSB medium until an OD₆₀₀ of 0.2 was reached. Cells were harvested by centrifugation, washed with 0.85% NaCl and, then suspended in 10 mL 0.1 M glycine. A 500 µL volume of 1 M HCl pH 2.2 was added to the suspension before gentle shaking (180 rpm) on ice for 30 min. Cells were removed by centrifugation and the supernatant mixed with 500 µL of Tris Base pH 7.0. The suspension was again shaken on ice for 30 min, and then centrifuged for 1 h at 18,000 rpm and 4 °C. The supernatant was then desalted on an Amicon column (Millipore) with a molecular weight cut-off of 30 kDa. The presence of concentrated VapA was confirmed by SDS-PAGE, which showed the presence of the protein with high purity.

Total amounts of AcrV and VapA proteins were measured with a Bradford assay [22]. Concentrations were adjusted with PBS and mixed in a 1:1 volume with an oil adjuvant (PharmaQ, Norway) to reach a final concentration of 3 µg protein per 50 µL of vaccine solution.

2.4. Vaccination

Prior to vaccination, fish were anesthetized using 50 mg/L buffered 3-aminobenzoic acid ethyl ester (MS-222, Argent Chemical Laboratories) and then individually injected intraperitoneally (i.p.) with 50 µL of vaccine or PBS (as a control). Immediately after injection, fish were placed back in the tanks. In the experiment with purified *A. salmonicida* rAcrV and VapA proteins, fish received a second vaccination dose 5 weeks after the first injection.

2.5. Challenge

Fish were challenged with the wt strain of *A. salmonicida* JF5054. In both experiments using bacterins, the challenge was performed 8 weeks after vaccination. Fish were anesthetized using 50 mg/L buffered 3-aminobenzoic acid ethyl ester and then i.p. injected with 5 × 10² CFU in 50 µL of PBS. Following vaccination with AcrV and VapA, trout were challenged by i.p. injection with 2 × 10² CFU in 50 µL of PBS 10 weeks after the first vaccination (5 weeks after the boost).

2.6. Mass spectrometry

To analyze the impact of the *ascV* deletion on the T3SS of *A. salmonicida*, 50 mL of TSB medium were inoculated with 10⁹ wt or Δ *ascV* strain and cultivated at 18 °C with aeration (160 rpm) in presence of protease inhibitor (Complete, Roche Diagnostics). Cells were harvested during the exponential growth (OD₆₀₀ = 1.5). Cells were washed in PBS before being mixed in a 1:1 volume with SDS loading buffer and heated at 100 °C for 5 min. Proteins were separated in non-adjacent wells (to avoid well to well contamination) on 15% acrylamide SDS-PAGE gels and stained with Coomassie. The gel (one lane for each condition) was completely sliced from the stacking gel to the buffer front in 20–25 bands for protein in-gel digestion and MS analysis as described elsewhere [23,24].

2.7. LC-MS/MS data interpretation

LC-MS/MS data interpretation was conducted using the current UniProtKB database release (2012.06) of all known *A. salmonicida* protein sequences. The peptide-matching score summation (PMSS) method of relative protein quantification was used. This is a label-free technique that assumes ideal scoring for proteins as the summative of the identification scores of their constituent peptides freed upon digestion. A higher score represents a more abundant protein [25]. For this, the EasyProt search algorithm [26] was used as previously described [27].

2.8. Bioinformatics and statistics

The tridimensional structures of *A. salmonicida* AcrV was modelled using the crystal structures of *Yersinia* LcrV (PDB ID:1R6F) as a template for the automated mode of SWISS-MODEL (<http://swissmodel.expasy.org>) and the assembly of LcrV subunits to represent the T3SS pentameric tip complex of *Yersinia* was made with SwissPdb Viewer [28] and reproduced as described previously [29].

The Chi-square (χ^2) test with Yates' correction was used to assess if the difference in the survival rate between the groups vaccinated with the wt or Δ *ascV* mutant strain was statistically significant.

3. Results and discussion

In a first assay, fish were vaccinated with the wt strain, the Δ *ascV* mutant or with PBS only (control group). Eight weeks later, all fish were challenged with 5 × 10² CFU/animal of wt *A. salmonicida*. Fish

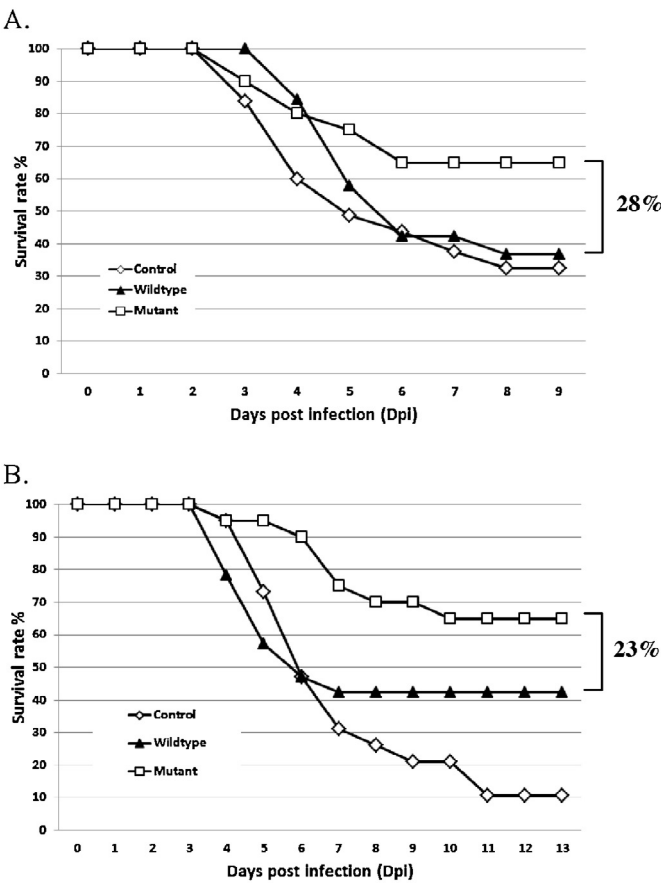


Fig. 1. Survival of fish vaccinated with bacterins and subsequently challenged with *A. salmonicida*. Panels A and B present the results of two independent vaccination experiments with bacterins (10⁷/fish) prepared from wild-type (virulent) and isogenic Δ *ascV* mutant strains of *A. salmonicida*. Control fish were injected with PBS only.

were monitored for 9 days post-infection (dpi) by which time the survival rate in the control group had plateaued at 32% (Fig. 1A). Vaccination with the inactivated wt strain brought no significant protection with 37% survival measured at 8 dpi (versus 32% for the control group) (Fig. 1A). In contrast, the group vaccinated with the Δ *ascV* mutant showed significant protection with a survival rate of 65% by day 6 post-inoculation (Fig. 1A).

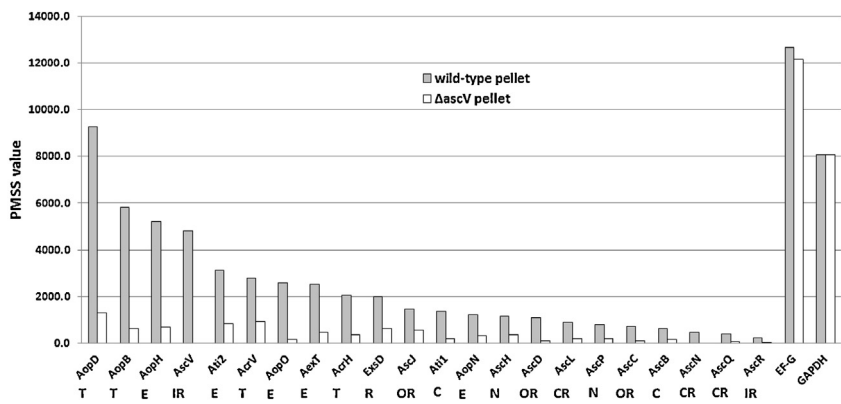


Fig. 2. Relative amounts of *A. salmonicida* T3SS proteins in wt and Δ *ascV* mutant pellets used to prepare bacterins. The histogram shows the amount of T3SS proteins in wt cell pellets (grey bars) versus the Δ *ascV* mutant cell pellets (white columns) used to prepare the bacterins. Structural roles of the T3SS proteins are given as follows: C = T3SS chaperone; CR = C-ring; E = effector; IR = inner membrane ring; N = needle; OR = outer membrane ring; R = regulator; T = translocator. Elongation factor G (EF-G) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are illustrated as reference values.

In a second, independent experiment using the same parameters, onset of mortality was on day 4 dpi in all 4 groups. By day 11 dpi, the survival rate in the control group was 10% whereas survival of fish vaccinated with the wt strain was 42% (Fig. 1B). The level of protection was highest in the group vaccinated with the deletion mutant $\Delta ascV$ at 65% survival. A fourth vaccination group that was vaccinated with a bacterin prepared from the $\Delta ascV$ mutant strain complemented *in trans* with the wt *ascV* gene [19] (*ascV/ascV⁺*) had a survival rate of 50% (data not shown). In both trials, vaccination with the $\Delta ascV$ mutant resulted in an improved survival rate in comparison to the group vaccinated with the wt strain (Fig. 1). A Chi-square test with Yates' correction (wt versus $\Delta ascV$ vaccination) was performed with the results from both experiments. This analysis revealed the statistic significance of the difference in the survival rate between the groups vaccinated with the wt or $\Delta ascV$ mutant strain ($\chi^2 = 4.559$ with 1 degrees of freedom, two-tailed P value = 0.0328). The results of these experiments were unexpected

and challenged the hypothesis that mounting specific antibodies against proteins of the T3SS yields in better protection.

In order to study the antigenic differences between the wt and the $\Delta ascV$ mutant, we performed a high-throughput semi-quantitative proteomic analysis of the major proteins present in cell-pellets of the wt and $\Delta ascV$ mutant strains, which were used to prepare the bacterins. The *ascV* deletion mutant had significantly reduced quantities of almost all components of the T3SS in comparison to the wt strain (Fig. 2 and Supplementary Table 1). Furthermore, the difference in amount of T3SS proteins was the principle difference between the two strains. Taken together, the results suggested that one or more components of the T3SS present in the wt strain may partially inhibit the establishment of an efficient immune response.

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2013.08.057>.

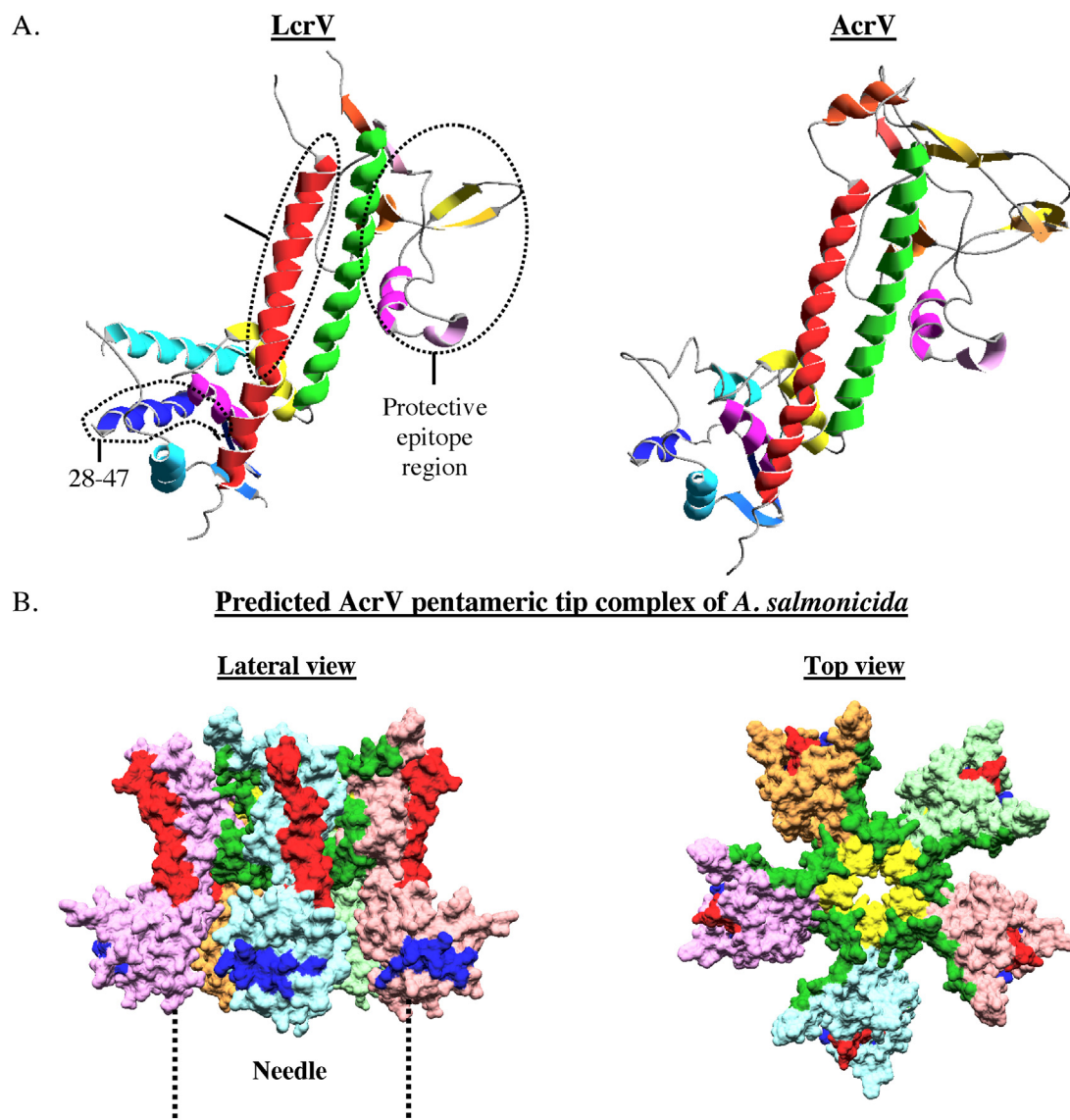


Fig. 3. Structural representation of *Yersinia* LcrV and *A. salmonicida* AcrV and the predicted pentameric AcrV tip complex of the T3SS in *A. salmonicida*. (A) Structural comparison of *Yersinia* LcrV and *A. salmonicida* AcrV. Amino acid residues in red and dark blue correspond to conserved regions associated to immunomodulation in LcrV (residues 28–47 and 271–300 in LcrV) [41,43]. (B) The predicted pentameric AcrV tip structure of *A. salmonicida* T3SS. Amino acid residues in red and dark blue correspond respectively to regions associated to immunomodulation in LcrV [41,43]. Amino acids in dark green represent the region corresponding to a major epitope recognized by LcrV polyclonal sera [49] and amino acid residues in yellow show the BA5 plague protective epitope of LcrV [50].

There are published observations that support this hypothesis. For example, AcrV and AopB homologues in *Yersinia* (LcrV and YopB) mediated immunosuppression when they are exposed to host immune cells [30,31]. Furthermore, homologues in other pathogenic bacteria of the effectors AopH [32], AexT [33], AopP [34], AopO [35] and AopN [36] and the regulator ExsB [37] are immunosuppressive when they are translocated or expressed directly in the host cells (short distance effect). These effectors are produced at strongly reduced amounts in the Δ ascV mutant strain (Fig. 2). However, it is not known if these T3SS proteins interact, like LcrV, with receptors of the innate immune system to play an immunomodulatory role when they are secreted into the extracellular environment of host cells (putative long distance effect) [30]. In this case, the lower amount of effectors/translocators in Δ ascV bacterins might also explain why the vaccination with the inactivated Δ ascV mutant brought a better protection than the inactivated wt strain.

The best described example of T3SS playing a role in disrupting the host defence is likely LcrV, which forms the tip of the T3SS needle and translocon subunit of *Yersinia*. This protein inhibits host inflammatory responses [38,39], amplified the release of IL-10 from host cells [30,40,41] and induces immunosuppression by promoting the differentiation of tolerogenic dendritic cells via the interaction with TLR2/TLR6 and CD14 receptors [42].

Two regions of LcrV at least were shown to be associated with the induction of the IL-10 immunomodulatory responses (Fig. 3A) [41,43]. *A. salmonicida* AcrV shows a high conservation with these fragments of LcrV (66% and 93% of similarity) and these are exposed on the outside of the predicted pentameric tip complex (Fig. 3B). We predicted that these conserved regions could play the same immunomodulatory function in AcrV. This assumption is supported by the observation that IL-10 levels were significantly down-regulated in mice infected with a Δ acrV mutant of *Aeromonas hydrophila* [44]. Thus, it may be possible that immunosuppressive AcrV present in our w.t. bacterin preparations (Fig. 2) interfered with vaccination against furunculosis.

In vitro, specific polyclonal antibodies against AcrV protect fish cells from the cytotoxicity induced by *A. salmonicida* [20] however, the protective effect of AcrV in fish vaccination is not known. To clarify this point, we immunized fish with either purified recombinant *A. salmonicida* AcrV (rAcrV) or purified VapA (control for a protective effect [13]) (Fig. 4).

While vaccination with VapA offered some degree of protection, vaccination with rAcrV showed no protection against challenge with the wt strain (Fig. 4). Similar observations have been made with the tip complex protein Bsp22 of *Bordetella bronchiseptica* [45] and BipD of *Burkholderia pseudomallei* for which vaccination with the recombinant protein brought no protection [46] while the vaccination with a live Δ bipD was partially protective [47]. Of note, the protective antigenic region of LcrV is conformational and found between amino acids 135 and 275 [48] (Fig. 3A) which only presents a weak similarity with AcrV. This domain corresponds to the entry of the T3SS pentameric tip complex (Fig. 3B).

4. Conclusions

Vaccination of rainbow trout with bacterins prepared from a wt, virulent strain and an isogenic T3SS mutant of *A. salmonicida* indicated the presence of T3SS proteins in the vaccine preparation decreased the level of protection against *A. salmonicida* infection. These results challenge the hypothesis that antibodies against the T3SS will provide better protection and demonstrate that further investigations are needed to better our understanding of immune responses against *A. salmonicida*. To this end, we recommend that future publications involving *A. salmonicida* bacterin vaccines describe how the vaccines are prepared and mention if the *A. salmonicida* strains used are virulent and express T3SSs.

Conflict of interest

None declared.

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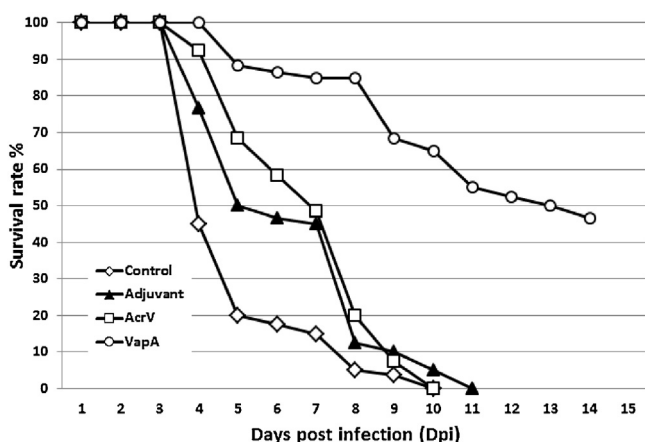


Fig. 4. Survival of fish following vaccination with purified proteins and subsequent challenge with *A. salmonicida*. Survival rates of fish were vaccinated with PBS (control), oil adjuvant only, rAcrV (3 µg/fish) + adjuvant or VapA suspension (3 µg/fish) + adjuvant are given. Fish were given booster doses 5 weeks after vaccination and challenged (2×10^2 CFU/animal) 10 weeks after vaccination.

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